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An intronic (A/U)GGG repeat enhances the splicing of an alternative intron of the chicken β-tropomyosin pre-mRNA

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ABSTRACT

Computer analysis of human intron sequences have revealed a 50 nucleotide (nt) GC-rich region downstream of the 5′ splice site; the trinucleotide GGG occurs almost four times as frequently as it would in a random sequence. The 5′ part of a β-tropomyosin intron exhibits six repetitions of the motif (A/U)GGG. In order to test whether these motifs play a role in the splicing process we have mutated some or all of them. Mutated RNAs show a lower *in vitro* splicing efficiency when compared with the wild-type, especially when all six motifs are mutated (>70% inhibition). Assembly of the spliceosome complex B and, to a lesser extent, of the pre-spliceosome complex A also appears to be strongly affected by this mutation. A 55 kDa protein within HeLa cell nuclear extract is efficiently cross-linked to the G-rich region. This protein is present in the splicing complexes and its cross-linking to the pre-mRNA requires the presence of one or several snRNP. Altogether our results suggest that the G-rich sequences present in the 5′ part of introns may act as an enhancer of the splicing reaction at the level of spliceosome assembly.

INTRODUCTION

The discovery of introns in the late 1970s rapidly raised the question of how does the cell manage to perform the excision of these intervening sequences, which may be up to 100 kb long, with such extreme precision. Examination of gene sequences has revealed the existence of conserved sequences at the 5′ and 3′ junctions of exons and introns (1). Many experiments have shown the involvement of these elements in the splicing reaction and their interactions with specific components of the splicing machinery (spliceosome). The spliceosome is made up of five small nuclear ribonucleoparticles and a number of known and still uncharacterized protein factors that assemble on the pre-mRNA following a well-defined pathway (2). However, the presence of the characterized consensus sequences may not be sufficient to explain the precise recognition of the splice sites, because similar sequences are often present in other positions on the pre-mRNA. Sometimes these cryptic sites match the consensus sequences better than the actual sites (3). Understanding how the splicing machinery distinguishes between genuine and cryptic sites mostly remains an open question. Recognition of the splice sites is an even more complicated question in cases of alternative splicing, where a site can be efficiently selected in a particular cell type and completely ignored in others (2,4). All these observations suggest that recognition of the actual sites and the eventual choice between them may require other *cis*-acting elements. During recent years various examples of such elements have been described, both in introns and in exons (for reviews see 2,5,6).

The search for *cis*-acting elements involved in splice site recognition has also been performed by computer analysis of pre-mRNA sequences of databanks. Such analysis has recently revealed that the first 50 nt of human introns is particularly rich in guanosine and cytosine residues (7). In particular, the trinucleotide GGG occurs almost four times as frequently as it would in a random sequence. This result suggests that these G-rich motifs may be involved in the splicing process.

One intron of the chicken β-tropomyosin pre-mRNA exhibits such GGG repetitions. The chicken β-tropomyosin pre-mRNA internal region contains two exons that are spliced in a mutually exclusive way (8). Exon 6B is specifically used in skeletal muscle cells and exon 6A is recognized in all other cell types. The intron that lies between exons 6B and 7 (IVS B7) contains a region of 60 nt that is particularly rich in guanosines. In the region that separates the 5′ splice site and the branch site the motif (A/U)GGG is repeated six times. We have performed systematic mutations of these different motifs to reveal their involvement for IVS B7 *in vitro* splicing. The decreased splicing efficiency produced by these mutations is particularly pronounced when all six motifs are mutated and this is correlated with inhibition of spliceosome assembly. In addition, UV cross-linking experiments have revealed a protein of ~55 kDa that is mainly linked to this region. This protein is present in the splicing complexes and its cross-linking to pre-mRNA depends on the presence of one or several snRNP. Altogether our results suggest that the G-rich

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sequences present in the 5′ part of introns may act as an enhancer of the splicing reaction at the level of spliceosome assembly.

MATERIALS AND METHODS

Plasmid constructions

All the constructs are derived from the pSma plasmid, which contains the *Eco*RI–*Hin*dIII fragment of the chicken β-tropomyosin gene spanning exon 6B to 7 (previously described in 9). To obtain the SΔ19 mutant the pSma plasmid was digested with *Bss*HII and *Ppu*MI and the major fragment filled in and re-ligated. The same fragment was ligated to a hybrid of oligonucleotides exhibiting cohesive ends to give the Sβ construct. The sequence introduced is 5′-CGATCACTTGTGTCAACACAG-3′ and it is the reverse version of a 5′ untranslated region (5′ UTR) of the human β-globin pre-mRNA. Systematic mutagenesis of the (A/U)GGG motifs (SRG mutants) was performed by standard oligonucleotide site-directed mutagenesis according to the Amersham handbook procedure. The s19 plasmid construct was achieved by a two-step subcloning from the pSma plasmid. First, the *Eco*RI–*Bss*HII fragment was removed and the vector was then filled in and re-ligated. In the second step the *Ppu*MI–*Hin*dIII fragment was removed and the plasmid was filled in and re-ligated to give the s19 construction. This plasmid and a control pSP65 vector were cleaved at the unique *Pvu*II site and gave RNA transcripts of 223 and 238 nt, respectively. The 3′BssHII and 3′PpuMI constructs were obtained by removing the *Eco*RI–*Bss*HII and the *Eco*RI–*Ppu*MI fragments of the pSma plasmid respectively. The plamids were then filled in and re-ligated.

In vitro transcription and splicing experiments

Radioactively labelled SP6 transcripts were synthesized as described previously with 5 μCi [α-^{32}P]UTP (650 Ci/mmol), 5 μCi [α-^{32}P]CTP (800 Ci/mmol), 5 μCi [α-^{32}P]GTP (800 Ci/mmol) (ICN) and 1 μg linearized plasmid (10). Reactions were incubated for 2 h at 37°C. RNA transcripts were then precipitated and gel purified. For splicing product analysis or for spliceosome assembly assays *in vitro* splicing reactions were performed as in Clouet-d'Orval *et al.* (9). Quantification were performed on a Molecular Dynamics PhosphorImager as described in Sirand-Pugnet *et al.* (11).

RNA–protein UV cross-linking

Splicing reactions were carried out as described above except that 0.3 pmol pre-mRNA were used and that PVA was omitted. In some experiments ATP was replaced by AMP-PCP, a non-hydrolysable analogue, and creatine phosphate was omitted. After cross-linking (254 nm, 15 min, 3000 μW/cm^2) RNase A was added to the samples (1.5 mg/ml final concentration). The material was incubated for 30 min at 30°C and then loaded onto a standard SDS protein gel. For UV cross-linking of purified complexes a 50 μl splicing reaction was carried out with 0.6 pmol pre-mRNA. Samples were loaded on a non-denaturing gel as described above. Slices containing the splicing complexes were irradiated as usual and subsequently submitted to RNase A treatment (1 mg/ml final concentration, 60 min incubation at 37°C). Slices were then incorporated into an SDS protein gel for the second dimension.

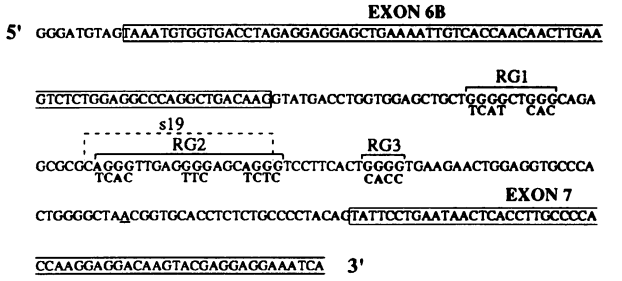

Figure 1. Sequence of the pSma construct. Exons sequences are written in boxes. RG regions are indicated. Nucleotide substitutions introduced in the SRG mutants are noted below the wild-type sequence. The sequence s19 shifted by 1 nt from the RG2 region is indicated with dashed lines. This sequence s19 has been deleted and substituted respectively in the SΔ19 and the Sβ mutants. The adenosine residue used as the branch site in the splicing reaction is underlined.

Immunoprecipitation assay

Cross-linked samples were incubated for 2 h at 4°C with an anti-Sm antibody (12) (generously provided by G.Cathala) pre-bound to protein A–Sepharose. After four washes with NET-2 buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% NP-40) immunoselected proteins were eluted with elution buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% NP-40, 0.8% SDS, 0.3 M sodium acetate, 0.3 μg/ml tRNA) and analysed on a SDS protein gel.

Micrococcal nuclease treatment of nuclear extract

HeLa cell nuclear extract was incubated for 30 min at 30°C with 1 mM $CaCl_2$ and 0.1 U/μl micrococcal nuclease. EGTA was then added to a final concentration of 2 mM and incubation was continued for a further 5 min. In a control extract EGTA was added together with $CaCl_2$ and micrococcal nuclease and incubated for 30 min at 30°C (13).

RESULTS

Mutations in the G-rich sequence of IVS B7 suggest a role in the splicing process

The intron that lies between exons 6B and 7 of chicken β-tropomyosin pre-mRNA contains a G-rich sequence located between the 5′ splice site and the branch site (Fig. 1). In this sequence the motif (A/U)GGG is present six times. These motifs are distributed in three regions, which we have named RG1, RG2 and RG3, which contain two, three and one copies of the motif, respectively. In order to test whether these motifs were involved in the IVS B7 excision process we first took advantage of the presence of two unique restriction sites that border the RG2 region. Deletion (SΔ19) and replacement of this 19 nt long region by a β-globin 5′ UTR-derived sequence (Sβ) was performed on the pSma plasmid (Fig. 1). Surprisingly, the two mutations have a very different effect on pre-mRNA splicing (Fig. 2). Deletion (SΔ19) has nearly no influence, but replacement (Sβ) decreases the splicing efficiency by >30% in comparison with the wild-type precursor. These apparently contradictory results lead us to think that the Sβ sequence might have an artifactual inhibitory effect.

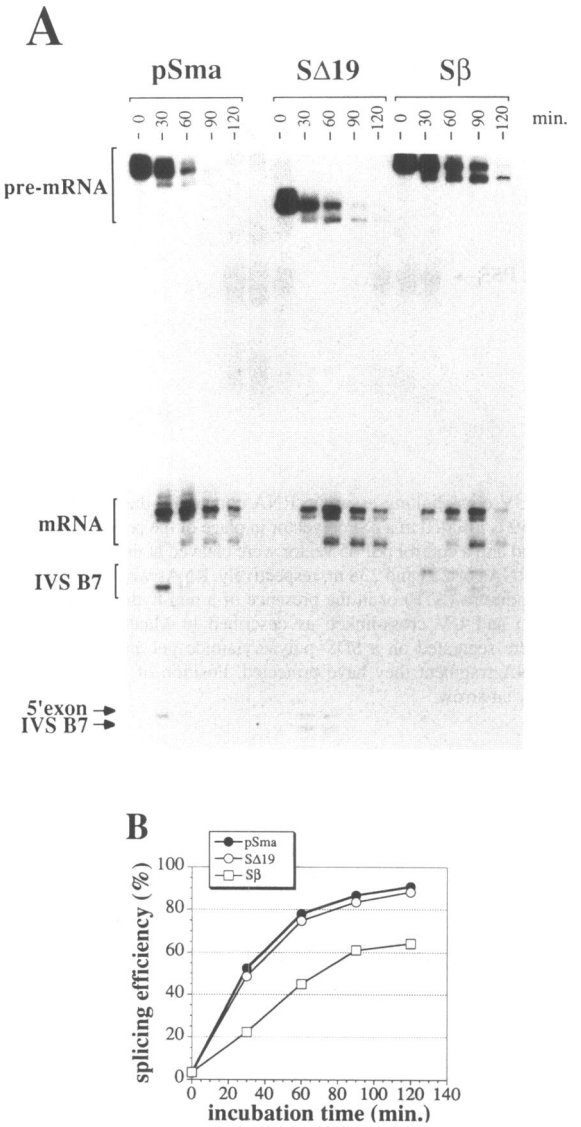

Figure 2. *In vitro* splicing reaction of pSma, SΔ19 and Sβ precursors. (**A**) Splicing products and pre-mRNA molecules were resolved on a denaturing gel. Positions of the pre-mRNA, mRNA, 5′ exon and final intron are indicated. The position of the final lariat is different for the three transcripts as a consequence of their different lenght. (**B**) Quantification of the splicing reactions. Precursor and messenger RNAs signals were quantified and the percentage of spliced product was calculated for each time.

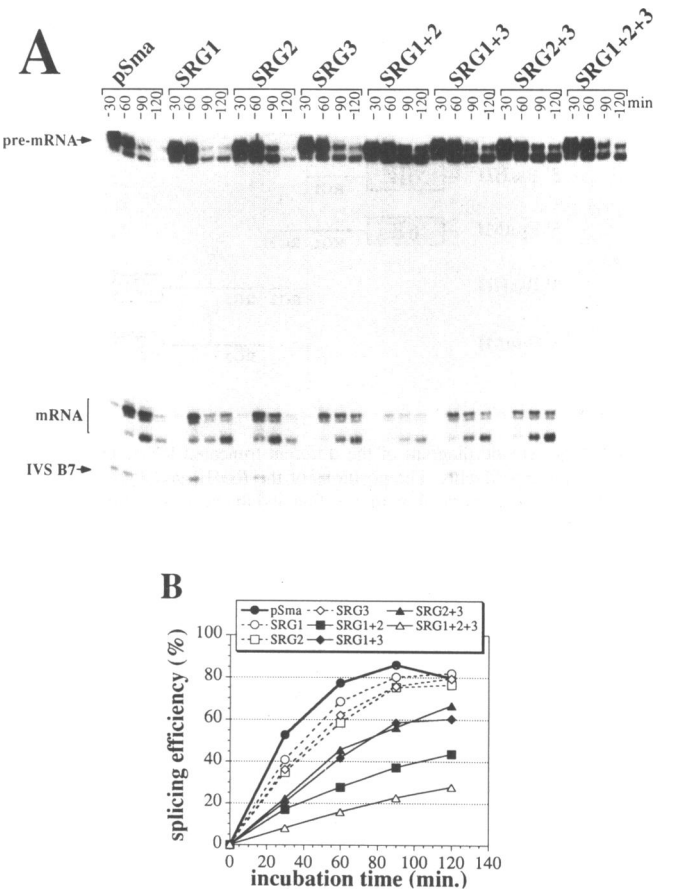

Figure 3. Influence of the (A/U)GGG motifs on the *in vitro* splicing of pSma pre-mRNA. (**A**) Time course of formation of the splicing products in a HeLa cell nuclear extract. Positions of the precursor and products RNAs are indicated. (**B**) Splicing efficiency of the different SRG mutants. Quantification of the pre-mRNA and mRNA signals was achieved and the splicing efficiency was calculated as the fraction of spliced product at each time point of the kinetic.

In order to test this possibility two other sequences were substituted to s19. These mutations also inhibit splicing (data not shown). These results suggest that the 19 nt that cover three copies of the (A/U)GGG motif can actually enhance splicing of exon 6B/7. One interesting hypothesis to explain the absence of effect of the deletion proposes a cooperative activation by the different RG regions. In this hypothesis deletion SΔ19 suppresses three motifs, but it also brings the regions RG1 and RG3 close to each other and thereby restores the cooperative activation. In contrast, substitution of the 19 nt eliminates the same number of motifs, but keep the distance between the RG1 and RG3 regions constant. The separation of the remaining RG regions would not allow cooperative activation of the splicing process.

Systematic mutation of the (A/U)GGG motifs reveals a cooperative activation of splicing

In order to confirm these results and to test if the motifs present in the RG1 and RG3 regions also activate IVS B7 splicing each of the three RG regions were mutated. In these mutants only the (A/U)GGG motif sequences have been modified (Fig. 1). Mutants containing a combination of these mutations were also constructed. Figure 3 shows that all the single region mutations have only a small effect; they decrease the splicing efficiency by 10–20%. The combination of two single mutations leads to a stronger inhibition (~40% for SRG1+3 and SRG2+3 and >60% for SRG1+2). The triple mutant is the most affected, with a decrease in splicing efficiency of ~75% compared with the wild-type.

Spliceosome assembly efficiency was compared between pSma and SRG1+2+3 pre-mRNAs using a non-denaturing gel assay. Compared with pSma, the proportion of pre-complex A and spliceosome B assembled on SRG1+2+3 are decreased by 30 and 80%, respectively (data not shown). This suggests that the (A/U)GGG motifs may be involved in several steps of spliceosome assembly.

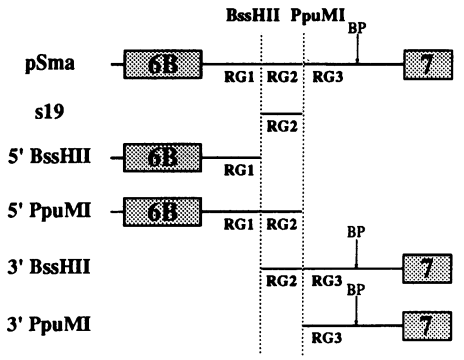

Figure 4. Schematic diagram of the different truncated RNAs used for UV cross-linking experiments. The positions of the *Bss*HII and *Ppu*MI cleavage sites are denoted. The branch point position and the three RG regions are also indicated.

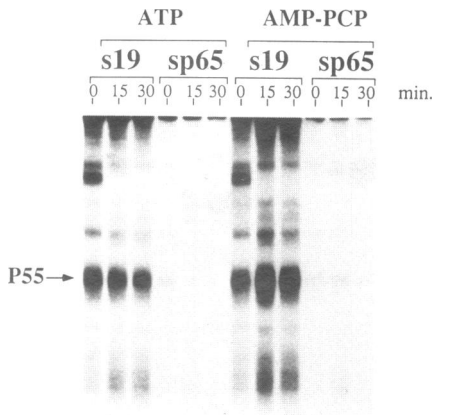

Figure 5. UV cross-linking assay on RNA containing the region RG2. The sequence s19 is inserted in a pSP65 vector in place of the polylinker sequence. This plasmid and a control pSP65 vector were cleaved at the pSP65 *Pvu*II site leading to RNAs of 223 and 238 nt, respectively. RNAs were incubated under splicing conditions (ATP) or in the presence of a non-hydrolysable analogue (AMP-PCP) and UV cross-linked as described in Material and Methods. Proteins were separated on a SDS–polyacrylamide gel and revealed by the labelled RNA fragment they have protected. Position of the P55 protein is indicated by an arrow.

Altogether these results indicate that the (A/U)GGG repetitions present in the 5′ part of the IVS B7 intron strongly enhance splicing. Moreover, the combination of mutations has a clearly stronger effect that the sum of the single region mutations, which suggests a cooperative effect between the different motifs. This last conclusion leads us to hypothesize that (A/U)GGG motifs may bind one or several splicing factors in a cooperative way.

UV cross-linking experiments reveal a protein of 55 kDa bound to the G-rich region of IVS B7

UV cross-linking assays were first performed on RNA s19 (223 nt), which contains the RG2 region of IVS B7, and a control RNA derived from a pSP65 plasmid (238 nt) (Fig. 4). The RNAs were incubated for different times under splicing conditions (ATP) or in the presence of a non-hydrolysable analogue (AMP-PCP) (Fig. 5). Although an equivalent number of pmol of s19 and sp65 transcripts was used in the experiment, protein signals were all very weak with the sp65 control RNA. One major band, corresponding to a protein of apparent molecular weight 55 kDa, was found cross-linked to the s19 RNA. Multiple bands corresponding to proteins of ~30 kDa were also present, as well as a protein of ~100 kDa that only appears at 0 min. These proteins were not studied further. The 55 kDa protein appears to be cross-linked specifically on RNA s19 with a high efficiency. We named this protein P55. This result suggests that the G-rich region of IVS B7 may enhance the splicing process through its capacity to bind P55 protein. Moreover, this protein is efficiently cross-linked at 0°C in the absence of ATP, which suggests a potential implication in the very first steps of spliceosome assembly.

In order to test whether P55 cross-linking also occurs in the context of longer RNA molecules several truncated constructs and the entire pSma precursor were used in an UV cross-linking assay (Fig. 4). The result is presented in Figure 6. P55 is cross-linked very efficiently to the entire pSma pre-mRNA, but very poorly on the 5′BssHII and the 3′PpuMI fragments, which contain the RG1 and the RG3 regions, respectively. Efficient cross-linking of P55 is only observed in molecules containing the RG2 region. Interestingly, the 5′PpuMI RNA (RG1+2) is a much better substrate than 3′BssHII RNA (RG2+3) for P55 cross-linking. This may suggest a better cooperative interaction between

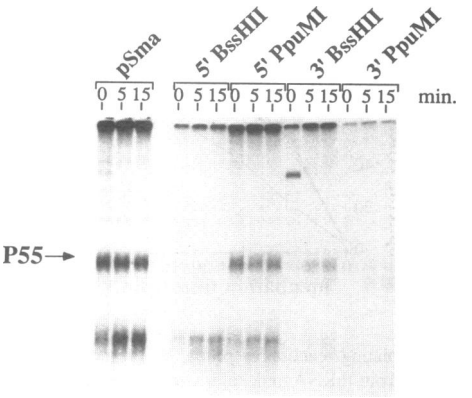

Figure 6. UV cross-linking experiment on truncated pSma RNAs. RNAs were incubated in nuclear extract in the presence of AMP-PCP for different times. Cross-linking was achieved as described in Material and Methods. Position of P55 is indicated by an arrow.

RG1 and RG2 than between RG2 and RG3 in binding P55. Alternatively, this may also suggest a requirement for a functional 5′ splice site for efficient binding of P55. However, this last hypothesis appears rather unlikely, because a point mutation that changes the consensus first G of IVS B7 into a C has almost no influence on P55 cross-linking (data not shown).

Finally, we performed UV cross-linking assays on several SRG mutant pre-mRNAs (Fig. 7). We observed a significant decrease in P55 signal for double and, especially, triple mutants. However, a strict correlation between the P55 cross-linking level and splicing efficiency cannot be made, since the mutant SRG1+2 exhibits a P55 signal equivalent or even slightly stronger than the

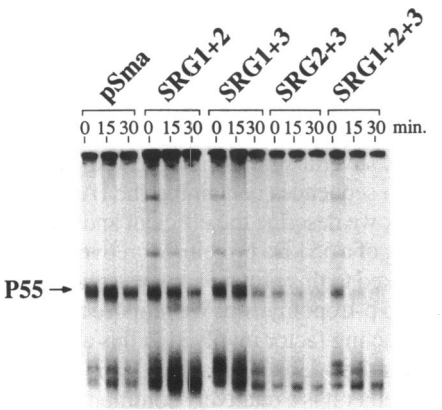

Figure 7. Influence of the (A/U)GGG motifs on P55 cross-linking. pSma, SRG1+2, SRG1+3, SRG2+3 and SRG1+2+3 pre-mRNAs were incubated with nuclear extract in the presence of ATP. At different times samples were submitted to UV cross-linking as described in Material and Methods.

other double mutants (SRG1+3 and SRG2+3), while it is spliced significantly less well than them.

P55 is part of the splicing complexes

In order to test whether the P55 interaction was related in some way to spliceosome assembly we used several approaches.

First, the presence of P55 in the splicing complexes was investigated by a two-step gel assay. The heterogenous H complex and the A and B splicing complexes were purified by non-denaturing gel electrophoresis. After irradiation and RNase A treatment of the slices proteins were separated on a SDS gel (Fig. 8A). Although the signal is lower than in the control, P55 appears to be present in all three complexes.

Second, P55 is immunoselected by an anti-Sm antibody (which immunoprecipitates all the snRNPs; 12) even though RNase A treatment, which degrades all unprotected pre-mRNA, is performed before the immunoselection (Fig. 8B). This suggests a relatively strong interaction between the cross-linked P55 and one or several snRNPs.

Finally, treatment of nuclear extract with micrococcal nuclease induces a dramatic decrease in P55 cross-linking (Fig. 8C). This indicates a noticeable relationship between P55 protein and the splicing machinery.

Altogether these results strongly suggest that P55 binding to pre-mRNA and spliceosome assembly are two closely related processes.

DISCUSSION

In the present work we investigated the involvement of a G-rich sequence in the splicing of the β-tropomyosin IVS B7 intron. We have shown that the six times repeated motif (A/U)GGG acts as an enhancer of the splicing process. One interesting question raised from this observation is the mechanism by which this *cis*-acting element influences the efficiency of splicing and, especially, spliceosome assembly. Our results indicate that mutation of the six motifs has a stronger effect than the sum of the single RG region mutations, which reveals a cooperative effect between the several copies of the motif. Such cooperativity of short repeated

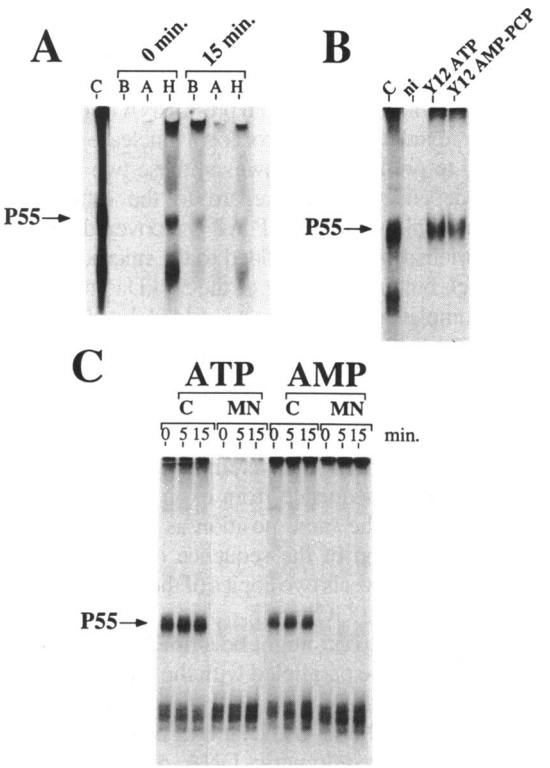

Figure 8. P55 and splicing complexes. (**A**) A non-denaturing gel assay was used to separate the H, A and B complexes assembled on the pSma precursor after 0 and 15 min reactions. The slices were cut out of the gel and cross-linked as described in Material and Methods. Slices at the positions of the A and B complexes were tested at 0 and 15 min, even though only the H complex was detectable at 0 min. C, a 0 min sample was submitted directly to the UV cross-linking procedure (without separation on the native gel) as a control. (**B**) Immunoselection of P55 with an anti-Sm antibody. After a standard splicing reaction (15 min, ±ATP), UV cross-linking and RNase A treatment samples were mixed with an anti-Sm antibody or with a non-immune serum (ni). Selected complexes were resolved on a SDS–polyacrylamide gel. C, control without immunoselection. (**C**) UV cross-linking assay on the pSma pre-mRNA incubated with a control (C) or a micrococcal nuclease-treated nuclear extract (MN). Position of the P55 protein is indicated by an arrow.

motifs in pre-mRNA splicing has already been described. In *Drosophila* the *tra* and *tra-2* gene products positively regulate usage of the female-specific 3′ splice site of *doublesex* (*dsx*) pre-mRNA by interacting with a 13 nt motif repeated six times in the 5′ part of the downstream female exon (14–19). Another example has been provided by study of the alternatively spliced exon EIIIB of rat fibronectin pre-mRNA. Cell-specific usage of the 5′ splice site of exon EIIIB is enhanced by a short motif, TGCATG, repeated eight times in the downstream intron (20). However, in both cases the sequences of the repeated motifs are different from the IVS B7 (A/U)GGG motifs and they probably act through a different mechanism.

The cooperative activation induced by IVS B7 G-rich motifs has encouraged us to look for a potential factor that may bind these motifs in a cooperative way. We have detected a 55 kDa protein (P55) that is very efficiently cross-linked to this region. P55 has several characteristics that make it a potential candidate for being the mediator of the G-rich sequence activating effect. This protein is present in the different splicing complexes and several motifs are required for efficient binding. Moreover, P55

cross-linking is dependent on the integrity of one or several snRNP. This last property is very reminiscent of work by Mayrand and Pederson showing that hnRNP A1 and an unidentified protein of 59 kDa are cross-linked to β-globin pre-mRNA (21). Pre-treatment of a nuclear extract with micrococcal nuclease abolishes their cross-linking to pre-mRNA. However, these two proteins exhibit a different dependence with regard to the different snRNPs. Efficient cross-linking of hnRNP A1 is recovered when a U1- or a U1+U2-enriched fraction is added to the micrococcal nuclease-treated extract, but cross-linking of the 59 kDa protein is restored only when complete nuclear extract is added. Interestingly, hnRNP A1 and the 59 kDa protein are efficiently cross-linked at 0°C and are present in the splicing complexes assembled on β-globin pre-mRNA. Knowing these results, an appealing hypothesis is that the 59 kDa protein and P55 may be identical. Consistent with this hypothesis is the fact that a cross-linking assay with a β-globin precursor-containing sequence from exon 1 to exon 2 reveals a protein migrating at the same position as P55 (data not shown). Moreover, examination of the sequence of the 5′ region of the β-globin first intron reveals two copies of the (A/U)GGG motif and four copies of an (A/U)GG motif.

The hypothesis that P55 might be a hnRNP or a hnRNP-associated protein has to be paralleled with the intriguing observation that some nuclear proteins, especially proteins identical or closely related to hnRNPs A1, A2-B1, D and E, can efficiently bind the $d(TTAGGG)_n$ human telomeric DNA repeat and even more tightly bind an oligoribonucleotide having r(UUAGGG) repeats (22). Interestingly, among them is an unknown protein of 55 kDa present in a fraction containing the hnRNPs A1 and A2-B1. This protein is also a good candidate for identity with P55. However, these proteins have been purified as a fraction of proteins able to bind the r(UUAGGG) motifs in a micrococcal nuclease-treated nuclear extract. This appears contradictory to our results, since P55 cross-linking to pSma pre-mRNA is highly dependent on the integrity of one or several snRNPs. Additionally, this result may be difficult to reconciliate with the fact that cross-linking of hnRNP A1 to β-globin pre-mRNA is dependent on the U1 snRNP (21). One possible explanation could be a sequence-dependent requirement of snRNPs for both hnRNP A1 and the 55 kDa protein. On some optimal specific sequences these proteins would bind with very good efficiency and this binding would be mostly independent of any other factor, but on sequences diverging in some degree from the optimal other *trans*-acting factors, such as snRNPs, may be required for efficient binding. Consistent with this hypothesis, binding of hnRNP A1 and the 55 kDa protein purified from a micrococcal nuclease-treated extract is abolished when a point mutation is introduced in any of the four first bases of the r(UUAGGG) motif (22). This suggests a highly specific binding to this particular sequence. In the case of hnRNP A1 the high specificity of binding is a somewhat controversial subject. Several studies employing ribonucleotide homopolymers and synthetic base analogues have argued against sequence-specific binding (23,24). On the other hand, much evidence indicates that hnRNP A1 can discriminate between RNA sequences (25–28). Among these last, a selection/amplification approach has identified a 20mer sequence that exhibits a very high affinity for purified bacterially expressed hnRNP A1 (28). Interestingly, this sequence is G-rich and contains two UAGGG(A/U) motifs closely related to the (A/U)GGG motif of IVS B7. Moreover, a UV cross-linking assay performed in a complete nuclear extract with this sequence used as substrate reveals not only hnRNP A1, but also an unidentified protein of 50 kDa. Finally, a recent study reports the characterization of two particular hnRNP proteins, hnRNP F and H, of 53 and 56 kDa, respectively. These proteins are highly related and display a particularly specific binding to poly(rG) *in vitro* (29).

In conclusion, several studies have described proteins of ~55 kDa that bind to sequences containing the (A/U)GGG motif. In the present work we describe the efficient and sequence-dependent cross-linking of a 55 kDa protein. Moreover, we show that the motifs cross-linked to this protein activate splicing of the IVS B7 intron of chicken β-tropomyosin. We propose that the P55 protein may be a *trans*-acting factor involved in this activation. Whether this (A/U)GGG motif-dependent activation is specific to the IVS B7 intron or whether it is a relatively common phenomena shared by many introns remains an open question. However, the G-rich motifs of IVS B7 chicken β-tropomyosin pre-mRNA are very conserved in other birds (quail and owl). Furthermore, in rat β-tropomyosin pre-mRNA this region is also very rich in guanosine residues and it contains seven AGGG motifs (Y.d'Aubenton Carafa, unpublished observations). Moreover, G-rich sequences have been described in the 5′ region of many human introns (7) and especially in short-sized GC-rich introns (Y.d'Aubenton Carafa, unpublished observations). These observations suggest that such G-rich motifs might be a general way of enhancing the splicing process, at least for a certain category of introns.

It is of interest to note that G-rich motifs closely related to the IVS B7 (A/U)GGG motifs we have found to be cross-linked to the P55 protein have also been shown to be a good target for hnRNP A1. This intriguing observation suggests that hnRNP A1 may also interact with the IVS B7 G-rich region. Many studies report that hnRNP A1 can modulate the choice between alternative 5′ splice sites, in combination with the factor ASF/SF2 (30–33). Interestingly, the ratio between these factors has been shown to influence the *in vitro* selection between the two mutually exclusive exons of rat β-tropomyosin pre-mRNA homologous to exons 6A and 6B (34). In the case of chicken β-tropomyosin IVS B7, which is spliced in a tissue-specific manner, one may hypothesize that hnRNP A1 and P55 can compete for binding of the the G-rich sequence and then be involved in the complex process that leads to the alternative use of the exon 6A and exon 6B 5′ splice sites.

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